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*Role of Membrane Environment and Membrane-Spanning Protein Regions in Assembly and Function of the Class II Major Histocompatibility Complex*

Ann M. Dixon<sup>†\*</sup> and Syamal Roy <sup>§</sup>

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**Abbreviated Title:** Role of Membrane Regions in MHC-II

<sup>†</sup> Department of Chemistry, University of Warwick, Coventry, CV4 7AL, UK.

<sup>§</sup> National Institute of Pharmaceutical Education and Research-Kolkata, 4 Raja SC, Mullick Road, Kolkata 700032, India.

\*To whom correspondence should be addressed: Dr Ann Dixon, Department of Chemistry, University of Warwick, Coventry, CV4 7AL, UK, Telephone: +44 2476 150037; FAX: +44 2476 524112; email: ann.dixon@warwick.ac.uk

## **Abstract**

Class II Major Histocompatibility complex (MHC-II) is a polymorphic heterodimer that binds antigen-derived peptides and presents them on the surface of antigen presenting cells. This mechanism of antigen presentation leads to recognition by CD4 T-cells and T-cell activation, making it a critical element of adaptive immune response. For this reason, the structural determinants of MHC-II function have been of great interest for the past 30 years, resulting in a robust structural understanding of the extracellular regions of the complex. However, the membrane-localized regions have also been strongly implicated in protein-protein and protein-lipid interactions that facilitate Class II assembly, transport and function, and it is these regions that are the focus of this review. Here we describe studies that reveal the strong and selective interactions between the transmembrane domains of the MHC  $\alpha$ ,  $\beta$  and invariant chains which, when altered, have broad reaching impacts on antigen presentation and Class II function. We also summarize work that clearly demonstrates the link between membrane lipid composition (particularly the presence of cholesterol) and MHC-II conformation, subsequent peptide binding, and downstream T-cell activation. We have integrated these studies into a comprehensive view of Class II transmembrane domain biology.

## **Keywords**

Class II Major Histocompatibility complex; transmembrane domain; membrane composition; membrane cholesterol; invariant chain

## Abbreviations

<b>MHC-II:</b>	Class II Major Histocompatibility complex
<b>HLA:</b>	human leukocyte antigen
<b>APC:</b>	antigen presenting cell
<b>PM:</b>	plasma membrane
<b>ER:</b>	endoplasmic reticulum
<b>Ii:</b>	Class II-associated invariant chain
<b>CLIP:</b>	class-II-associated invariant chain peptide
<b>TCR:</b>	T-cell receptor
<b>TMD:</b>	transmembrane domain
<b>FTIR:</b>	Fourier transform infrared
<b>MD:</b>	molecular dynamics
<b>BCR:</b>	B cell receptor
<b>p-MHC II:</b>	peptide-Class II complex
<b>CRAC:</b>	cholesterol recognition/interaction amino acid consensus sequence
<b>CCM:</b>	cholesterol consensus motif
<b>LD:</b>	Leishmania donovani
<b>SPR:</b>	surface plasmon resonance
<b>mAb:</b>	monoclonal antibody
<b>DPPC:</b>	1,2 dipalmitoyl-sn-glycero-3-phosphocholine
<b>DPC:</b>	n-dodecylphosphocholine
<b>PBD:</b>	peptide binding domain
<b>LACK:</b>	Leishmania homolog for the receptor of activated C Kinase

## Introduction

The Class II Major Histocompatibility complex (MHC-II), encoded by the human leukocyte antigen (HLA) gene complex in humans, is a polymorphic heterodimer composed of two type I membrane glycoproteins, known as the alpha ( $\alpha$ ) chain and the beta ( $\beta$ ) chain. The  $\alpha$  and  $\beta$  chains of MHC-II are encoded by three different loci in humans to produce three different types of Class II complex termed HLA-DR, HLA-DQ, and HLA-DP. These proteins have roughly 70% sequence similarity to one another, but display varying rates of polymorphism to generate an exceptional number of alleles. Infectious disease is thought to act as one of the main driving forces for MHC polymorphism, promoting a large diversity of MHC proteins in the population.

MHC-II binds antigen-derived peptides produced via protease or hydrolase degradation of exogenous proteins, and presents them on the surface of specialized antigen presenting cells (APCs) such as B cells, macrophages, and dendritic cells. MHC-facilitated peptide presentation on the surface of APCs leads to recognition by CD4 T-cells, subsequent T-cell activation, and elicitation of an immune response. Failure of Class II-directed T-cell activation, either via mutation to create a large number of known alleles or severe up- or down-regulation of Class II molecules, is implicated in autoimmune diseases such as rheumatoid arthritis[1], type I diabetes[2], and multiple sclerosis[2], as well as in HIV[3], asthma[4], certain cancers[5], and infectious diseases[6]. These examples highlight the importance of correct transport of peptide-loaded Class II molecules to the surface of APCs.

### *Current models of MHC-II Assembly*

MHC-II is transported to the plasma membrane (PM) from the site of biosynthesis in the endoplasmic reticulum (ER) via the endosomal transport pathway[7, 8]. This transport is initiated by a chaperone protein called the MHC Class II-associated invariant chain (Ii, also known as the Class II gamma chain or CD74), which is a homotrimeric type II membrane protein. In the classical model of Class II assembly and transport, it is thought that Ii trimers bind three MHC-II alpha/beta heterodimers ( $\alpha/\beta$ , Figure 1A) to form a nine-chain  $Ii_3(\alpha/\beta)_3$  complex. Formation of this complex involves direct interaction of the CLIP (class-II-associated invariant chain peptide, residues 81-104) region in Ii with the Class II peptide binding cleft, blocking binding of self-peptides [9]. Class II binding also shields the R-X-R ER retention motif present on the N-terminus of Ii. It has been shown that the cytoplasmic tail of the Class II  $\beta$ -chain is specifically required to overcome ER retention[10], however the length of this tail can be as short as three amino acids (ruling out steric hindrance as a likely mechanism)[11].

The nonameric model has been challenged recently by Koch and coworkers, who report evidence that each Ii trimer binds a single  $\alpha/\beta$  heterodimer to form a  $Ii_3\alpha/\beta$  pentamer (Figure 1B)[12]. Regardless of the stoichiometry, it is well-accepted that correct transport of the MHC-II from the ER to the PM requires binding of Ii, which directs the complex either to early endosomes or to the PM where it is internalized into clathrin-coated pits before reaching the MHC-II-rich compartment (MIIC)[7]. Ii is eventually degraded by proteases in endosomal compartments before Class II is loaded with antigenic peptide[13, 14].

The structural determinants of Class II Ii/ $\alpha/\beta$  complex formation have been the subject of great interest for the past three decades. It has been shown that formation of the Ii trimer is stabilized through protein-protein interactions in its C-terminal (luminal) domain involving residues 118-192, and that this region forms stable trimers in isolation[15]. Nuclear magnetic resonance (NMR) structural data were used to confirm the trimeric structure of a protein derived from this region[16], and by the late 1990s this region became known as the “trimerization” domain. Deletion of this luminal portion of Ii was shown to diminish trimer formation and endosomal targeting [17-19]. Structural and biochemical evidence for direct interactions between the Ii CLIP region and the  $\alpha/\beta$  peptide-binding groove has also been reported [20-27], and this interaction has been suggested as a major contributor to formation of the Ii/ $\alpha/\beta$  complex. Other sites of interaction between Ii and the  $\alpha/\beta$  complex have been proposed to lie outside of this CLIP region [15, 28, 29]. Wilson and coworkers demonstrated that Ii could associate with Class II molecules in which the peptide binding groove was already occupied, supporting the presence of other Ii-Class II interaction sites [29]. Park and coworkers also reported a direct interaction between the C-terminal trimerization domain and MHCII molecules[15].

The structural basis for MHC-II  $\alpha/\beta$  complex formation and peptide binding has also been investigated by a wide variety of methods [30-35]. It has been shown that the N-terminal domains in both chains pack together in a manner closely resembling the packing in Class I MHC in order to stabilize formation of the heterodimer and engage the T-cell receptor (TCR). These domains are highly polymorphic, and thus regulate the correct pairing of allelically-matched MHC-II

chains. The peptide binding groove lies in the amino-terminal domain and is formed by antiparallel  $\beta$ -sheets and antiparallel  $\alpha$ -helices, again closely resembling the arrangement in Class I MHC[30]. It has also been proposed that the  $\alpha/\beta$  heterodimers have an affinity to form dimers themselves, and this *dimer of dimers* is thought to increase the affinity for the CD4 co-receptor as well as play a role in T-cell response by facilitating TCR crosslinking[30, 36].

The studies above, along with many others, have resulted in a strong structural understanding of the intracellular and extracellular regions of Ii and MHC-II proteins. However, the membrane-localized regions of the complex have also been strongly implicated in MHC-II assembly, transport and function for the past thirty years. While these regions are inherently more challenging to characterize, they have been shown as sites of key protein-protein interactions involving the transmembrane domains of Ii,  $\alpha$ , and  $\beta$ . Indeed, it is highly likely that transfer of conformational information from the transmembrane regions to the extracellular domains could be transmitted by the connecting peptides that link the two. Membrane-localized regions are also sites of critical protein-lipid interactions, as evidenced by the sensitivity of MHC-II protein conformation (and subsequent T-cell activation) to changes in membrane environment. The accumulated evidence that these regions are sites of critical interactions is compelling, and should not be ignored when considering the functional mechanisms of MHC-II. This review describes work that forms the basis for our understanding of the role of the membrane and membrane-localized protein regions in assembly, transport and function of MHC-II.



## II. Discussion

### IIA. Membrane spanning protein regions are key interaction sites for assembly of the MHC-II throughout its transport pathway

*Assembly and function of Ii is influenced by transmembrane domain interactions.*

In 1999, Ashman and Miller reported the first evidence that the Ii transmembrane domain (TMD) is the site of critical protein-protein interactions that stabilize the functional Ii homotrimer [37]. This study demonstrated that residues 1-80 of Ii, encompassing the N-terminal cytoplasmic and transmembrane domains (see Figure 1B), could support trimerization in the absence of the luminal “trimerization” domain. In the same study, the role of the TMD was further explored by mutagenesis of a cluster of highly conserved polar amino acids (Gln47, Thr49, and Thr50, Figure 1C) in the center of the TMD which, when simultaneously mutated to hydrophobic residues (specifically Ala or Ile), prevented trimerization of the full-length Ii both *in vitro* and *in vivo*. This triple mutation also eliminated MHC-II binding to Ii, and thus prevented formation of the mature MHC-II complex and antigen presentation. The lack of trimer formation for the hydrophobic mutant was surprising since the luminal “trimerization” domain, confirmed to form trimers in the absence of the cytoplasmic and TMD [16], was intact in this construct. The authors suggested that disruption of the TMD interactions *in vivo* was likely altering the orientation of Ii with respect to the bilayer, either via tilting of the TMD to accommodate hydrophobic mismatch or

via membrane deformation. Such TMD rearrangement could then impact the orientation of the luminal domains of Li with respect to one another, thus weakening (or preventing) interactions in the soluble trimerization domain. The authors also suggest that the ability of soluble, recombinant Li to form trimers could reflect the high concentrations of the protein in these structural studies, typically in excess of 100-1000-fold greater than the concentration of Li in the ER. Indeed, overexpression of their Li TMD mutant to high concentration was able to rescue some degree of trimer formation, likely via alternative sites of interaction. Therefore, while structural studies of soluble domains provide very useful data, we suggest that these studies do not rule out other domains (such as the TMD) as sites of critical interaction.

Ashman's results were later expanded using biophysical and computational methods to determine the molecular basis of Li TMD interactions. In 2002, the TMD was investigated in isolation from the rest of the protein to probe the secondary structure of this region and propose a molecular model of its self-association[38]. In this study, Kukol and coworkers utilized Fourier transform infrared (FTIR) data obtained from  $^{13}\text{C} = ^{18}\text{O}$  labeled TMD peptides in conjunction with molecular dynamics (MD) simulation to resolve the backbone structure of the Li TMD helix and suggest potential sites of helix-helix interaction. They presented a structure of the Li TMD as a left-handed coiled-coil trimer stabilized by putative interhelical contacts involving Gln47 and Thr50, strongly supporting the work of Ashman and Miller described above [37]. In a separate study in 2006, we investigated the assembly and stoichiometry of the wild-type Li TMD in a variety of detergent conditions [39]. This study provided the first experimental evidence that the predominant oligomeric state

for the Ii TMD is a trimer with a dissociation constant of approximately 120 nM in n-dodecylphosphocholine (DPC) micelles. This low  $K_d$  suggests a very strong interaction and a clear bias toward Ii trimer formation, reflecting the biologically active oligomeric state of the full-length protein. Indeed, such a low  $K_d$  is unsurprising given that in vivo cross-linking of full length Ii at physiologically relevant concentrations revealed almost exclusive formation of Ii trimers and no detectable concentration of either monomeric or dimeric Ii species [37]. In the same study, we measured the effects of point mutation of Gln47, Thr49, or Thr50 on Ii TMD helix-helix interactions in a natural membrane bilayer, demonstrating that these interactions were facilitated in large part through interhelical H-bonding of Gln47 and Thr50 [39]. Taken together, these studies support a model for MHCII complex assembly that accounts for strong and specific Ii TMD interactions that may (i) initiate trimer formation (Figure 2A) and (ii) direct the orientation of the luminal domains for optimal interaction.

While the studies mentioned above implicate the cluster of TMD polar residues in Ii trimer formation, MHC-II binding and antigen presentation, a 2001 study presented conflicting data [40]. In this work, the entire C-terminal half of the Ii TMD (residues 42-53) was replaced with 12×Ala residues, thus removing the cluster of polar residues altogether. This substitution did not prevent ER translocation, Ii trimer formation, or association with Class II molecules. Instead, the mutant Ii associated with Class II molecules more strongly than the wild-type protein, probably reflected in part by its increased resistance to proteolysis. While these conflicting results are difficult to explain, one possible explanation may lie in the intermediate hydrophobicity of Ala which, in a mutant containing 12×Ala residues, leads to a

marked increase in the predicted free energy of membrane insertion as compared to the wild-type sequence [41]. A subtle increase in the free energy of insertion may alter properties such as tilt angle or TMD length, while more significant increases in insertion free energy will prevent membrane insertion of a sequence altogether. We suggest that the nature of the mutation in this work may substantially alter Ii-membrane interactions and this may account for differences observed.

#### *Engagement of Ii with Class II molecules via TMD interactions*

The polar residues in the Ii TMD that drive Ii trimer formation have also been reported to impact interactions between Ii and MHC-II molecules [37], and raise the question of whether the TMDs of all three proteins are sites of stabilizing interactions in the Ii-MHC-II complex (Figure 2B). In 2001, Castellino and coworkers [42] reported interaction between MHC-II and Ii regions outside of the CLIP domain in order to explain the efficient binding of the non-polymorphic Ii with a diverse range of MHC-II molecules displaying large differences in affinity for the CLIP peptide. In order to completely block possible interactions with CLIP, an antigenic peptide was covalently bound to the MHC  $\beta$  chain. Despite this, Ii was found to directly associate with MHC-II in detergent micelles. Truncation mutants were used to locate the site of MHC-II-binding within Ii, and it was traced to the TMD. An Ii construct containing only residues 28-58, mapping to the TMD, was shown to stably associate with MHC-II via interactions that lay well outside of the CLIP region. Since then, others have also reported the stable binding of Ii to MHC-II molecules in vivo via interactions in its cytosolic and transmembrane domains[43]. For example, a membrane-proximal YFR

motif in the cytoplasmic tail of the MHC-II  $\beta$  chain has been shown to regulate the activity of the ER-retention motif in Ii, likely through direct protein-protein interactions, between the  $\beta$ -chain and Ii. Expanding into a model that accounts for the above reports, we suggest that interactions localized to the TMDs of Ii and MHC-II molecules (either individually or as the preformed heterodimer) contribute to complex formation (Figure 2B), possibly even initiating complex formation whilst the CLIP and trimerization domains access their optimal binding arrangements. Thus far, there have been no reports of the molecular details of this interaction, however in Figure 1C we have mapped the residues in the Ii TMD that lie on the same helical face as Q47 and T50 and are thus likely to be excluded from a Ii-MHC-II interaction. The remaining Ii TMD residues would be available to direct Ii-MHC-II interactions, but it remains to be determined which residues are involved.

*TMD interactions impact MHC-II assembly, conformation, transport, and peptide-binding*

In 1984, Travers and co-workers [44] were the first to report the potential role of the MHC  $\alpha$  and  $\beta$  TMDs in MHC-II complex assembly and peptide binding. In this study, it was shown that conserved Gly residues in the TMDs of both MHC-II antigens are spaced such that they would map to a discrete face of an  $\alpha$ -helix, occupying positions 1 and 4 in a heptad repeat pattern typical for  $\alpha$ -helical coiled coils (see Figure 3A). A model was proposed in which helical MHC  $\alpha$  and  $\beta$  TMDs packed together along these Gly-containing faces. In this model, a Cys residue near the N-terminus of the  $\alpha$  TMD also lies on this Gly-containing interface (Figures 3A-B). This

Cys is very highly conserved, indeed it is invariant down to sharks [45] and is likely to play a key, yet currently unknown, role in Class II structure, function, or both. It has also been proposed that the  $\alpha$  and  $\beta$  TMDs are oriented by a salt bridge between a Glu residue in the  $\alpha$  chain and a Lys in the  $\beta$  chain [44, 46], as shown in Figures 3A-B. However the Glu residue in the  $\alpha$  chain is not conserved outside of mammals [45]. One hypothesis is that, in complexes missing this Glu, the interaction could be rescued via polar interaction involving the invariant Cys.

In 1992, Cosson and Bonifacino replaced the TMD in each MHC-II protein with that from the interleukin-2 receptor [46]. The resulting chimeras assembled incorrectly in COS cells, failing to bind conformation-dependent antibodies (i.e. antibodies that recognize only appropriately folded conformers of class II molecules) and being largely retained in the ER. In addition to replacing the entire TMD, mutants were constructed in which Gly residues present in both the MHC  $\alpha$  and  $\beta$  TMDs (Figure 3A) were collectively replaced with Val residues. This was one of the earliest reports experimentally demonstrating the role of the G-XXX-G motif, a now well-accepted helix-helix interaction motif [47-49], in stabilizing interactions between  $\alpha$  and  $\beta$  TMDs. Removing the TMD Gly residues had the same impact as replacing the entire TMD with that of an unrelated protein: the chains were unable to bind conformational antibodies and were retained in the ER. It was also shown that the MHC  $\alpha$  and  $\beta$  TMDs could drive association of other protein sequences into which they were grafted. This study supported the model first proposed by Travers and co-workers [44] and was a pioneering example of how the TMDs of MHC-II proteins could impact assembly and transport of the full-length protein. Subsequent reports

strengthened this conclusion, demonstrating that other types of changes to MHC-II TMD sequences also result in dysregulation of transport in vivo (although there have been reports contesting the role of the TMDs as well [50]). Truncation of the MHC  $\alpha$  and  $\beta$  chains to remove the cytoplasmic domains of both proteins and the C-terminus of the MHC  $\beta$  TMD lead to a large reduction of MHC-II expression on the plasma membrane [51]. Replacement of the MHC $\beta$  TMD with that of the  $\alpha$  chain lead to accumulation of MHC-II molecules in the early- and late-endocytic compartments[52]. In both studies, this impaired transport was not due to lack of association of Ii, as immunoblotting indicated Ii binding to mutant complexes (likely via interactions in the CLIP and luminal domains which were intact in all constructs tested). The changes in the TMD were instead thought to impact the global MHC-II conformation, leading to a transport-impaired conformation.

Sherman and coworkers also reported the far-reaching influence of  $\alpha$  and  $\beta$  TMD interactions on MHC-II conformation in their investigation of the impact of pH on peptide binding of purified versus membrane-bound MHC-II [53]. While purified, detergent solubilized MHC-II showed very little sensitivity to pH with regards to structural stability and peptide-binding properties, membrane-bound MHC-II on the cell's surface demonstrated large fluctuations in peptide binding with changes in pH. The results were attributed to the influence of TMD-lipid bilayer interactions on the conformation and/or flexibility of the N-terminal domains, which then modulated peptide binding. Although the nature of these conformational changes remains to be characterized in full, this study provides clear evidence that truncated, soluble class II proteins can show markedly different behavior to their membrane-bound (and

more physiologically-relevant) counterparts, and studies in which the membrane-localized proximal regions are deleted should be interpreted with caution.

The majority of work implicating MHC-II TMDs has been performed *in vivo* on full-length proteins. More recently, we have demonstrated *in vitro* that the TMDs of MHC-II molecules in isolation have a strong propensity to associate into homodimers and  $\alpha/\beta$  heterodimers in both model and natural membranes [54]. The results from this study indicated that interactions between the  $\alpha$  and  $\beta$  TMDs are strong and sequence-dependent, and validate the role of transmembrane Gly residues first suggested 26 years earlier by Travers and co-workers [44]. Our study also revealed, via mutagenesis and computational modeling, that there are two likely modes of interaction between the  $\alpha$  and  $\beta$  TMDs involving different Gly packing arrangements. The two Gly packing arrangements involved pairing of the single G-XXX-G motif in the  $\beta$  TMD (indicated by a bracket in Figure 3A) with either of two available G-XXX-G motifs in the  $\alpha$  TMD. The mutants from this work were introduced into the  $\alpha$  and  $\beta$  TMDs of mouse class II molecules, followed by *in vivo* measurement of MHC-II conformation and membrane localization in HEK293T cells [55]. The results revealed that the differential pairing of the G-XXX-G motifs directed the formation of two distinct MHC-II conformers (Figure 3B) in the ER, most likely under the guidance of Ii. Pairing of the sole  $\beta$  G-XXX-G motif with the C-terminal G-XXX-G motif in the  $\alpha$  TMD yielded a more robust interaction, but resulted in a MHC-II conformer that displayed weak T cell activation. Pairing via the N-terminal G-XXX-G motif in the  $\alpha$  TMD lead to formation of an I-A<sup>k</sup> MHC-II conformer, Ia.2<sup>+</sup>, previously shown to localize to lipid rafts, control B-cell calcium signaling, and enhance antigen



presentation to T cells [56, 57]. Lipid raft localization may be further facilitated by palmitoylation of the invariant Cys residue in the MHC-II  $\alpha$ -chain, shown in Figure 3. Palmitoylation of transmembrane and juxtamembrane Cys residues has been reported as a lipid raft-targeting mechanism for integral membrane proteins [58], and this has been demonstrated for I-A and HLA-DR complexes [59, 60].

The above studies present highly compatible models of the TMD interactions in MHC-II, whereby Gly residues pack together (either as discrete G-XXX-G motifs or as part of heptad repeat motifs) to stabilize complex formation. Indeed, a recent review highlighted known disease-associated MHC-II polymorphisms that target these Gly-rich helical TMD faces, and thus may significantly impair protein interactions and destabilize MHC-II complexes [61]. These complexes may be localized to specific membrane microdomains by palmitoylation of the highly-conserved Cys residue present at the transmembrane / connecting-peptide interface in the  $\alpha$  chain. Recently, the MHC-II Ii.2<sup>+</sup> conformer has also been shown to interact selectively with antigen-B cell receptor (BCR) complexes, becoming selectively loaded with BCR-derived peptide antigen prior to its expression on the cell's surface [62], and these interactions are thought to involve the TMDs of MHC-II and the BCR. MHC-II TMDs have also been implicated in interactions with MARCH family ubiquitin ligases [63] and tetraspanin proteins including CD82 [64-66], however the molecular determinants of those interactions have yet to be determined.

Taken together, the work described above demonstrates the strength and influence of TMD interactions in functional assembly of the MHC-II heterodimer, and

this has now been accounted for in the extended model we propose here for MHC-II assembly (see Figure 2C).

## **IIB. Membrane composition and membrane lipid environment impact MHC-II localization, conformation and function**

### *Lipid Rafts and localization of MHC-II protein.*

In addition to membrane-localized protein interactions, there is much evidence that the MHC-II is regulated by the properties of the membrane. The cell membrane of mammalian nucleated cells contains a variety of biological molecules, notably lipids (glycolipids, sphingolipids and cholesterol) and proteins. A dynamic clustering of sphingolipids and cholesterol allows the formation of specialized structures in the membrane known as “rafts” which are approximately 4-200 nm in diameter [67]. Biochemically, rafts are loosely defined as detergent resistant membrane microdomains based on their relative insolubility in mild non-ionic detergents at low temperature [68], although there is some controversy about their nature in *in vivo* conditions [69]. A number of membrane proteins are proposed to associate specifically with lipid rafts. Treatment of cells with cholesterol-binding pharmacological mediators perturbs the raft assembly and disperses raft associated proteins [70]. Several studies also demonstrate that a substantial fraction of MHC-II proteins are raft-associated, however the relative percentage of raft-associated protein reported in the literature ranges from roughly 50% [71] down to 10-20% [56, 72].

### *Importance of rafts in MHC-II–restricted T-cell activation*

Stimulation of T-cells by antigenic peptides presented in the context of MHC-II molecules requires a stable contact between T-cells and antigen presenting cells (APCs) for effective T-cell activation [73]. Lipid rafts in the outer leaflet of the membrane bilayer induce clustering of the peptide-MHC-II (p-MHCII) complex on the APC surface to increase the surface density of the p-MHCII, resulting in appropriate T-cell activation [74-76]. Disruption of lipid rafts by sequestration of cholesterol after treatment of APCs with methyl- $\beta$ -cyclodextrin impaired the presentation of p-MHCII complexes to T-cells [74]. However, such defects could be reversed at high cognate peptide concentrations, indicating that lipid rafts indeed play an important role in enhancing the T cell response at low ligand concentrations [77].

### *MHC-II TMD interacts with membrane cholesterol*

Direct interactions between membrane cholesterol and membrane-embedded protein regions can impact the biological function of membrane bound receptors such as cholecystokinin (type B), oxytocin, and nicotinic acetylcholine [78]. The nicotinic acetylcholine receptor has been shown to have internal sites capable of forming adducts with cholesterol, resulting in stabilization of the protein's structure [79]. These proteins bind cholesterol via a range of cholesterol binding sequence motifs found within their TMDs. The cholesterol recognition/interaction amino acid consensus sequence, generally referred as the CRAC motif, is a short, linear sequence that can be depicted as (L/V)-X1-5-(Y)-X1-5-(K/R). This motif is composed of a branched apolar Leu or Val residue, followed by a segment containing 1-5 of any

residues, an aromatic Y residue, a second segment containing 1–5 of any residue, and finally a basic Lys or Arg residue. The CARC motif, thus far only described for the human nicotinic acetylcholine receptor, is an inverted CRAC motif with the consensus sequence (K/R)X1–5(Y/F)X1–5(L/V). In the CCM (cholesterol consensus motif) [80] the amino acids interacting with cholesterol are not a linear sequence motif, but are distributed between TMDs within polytopic membrane proteins. One helical TMD contains the sequence motif (W/Y)(I/V/L)(K/R), whereby all residues must face the same side of the helix. In addition, another aromatic amino acid, either phenylalanine or tyrosine, is needed on a second helix to bind cholesterol from the other side. Both oxytocin and serotonin 1A receptors contain a strict cholesterol consensus motif (CCM), and in both there is a dramatic increase in agonist affinity in the presence of cholesterol [81, 82]. As highlighted in Figure 3A, analysis of the amino acid sequences of the mouse MHC-II TMDs revealed a putative CRAC-like motif at the C-terminus of the  $\alpha$  TMD [I/V-(X)4-F-(X)5-R]. Similarly, an overlapping CRAC/CCM-like motif [F-(X)2-L-(X)2-F-(X)1,3-R/R] was observed in the mouse MHC-II  $\beta$  TMD [83]. Comparison of these sequences to those from human and rat MHC-II suggests that these motifs are largely conserved in the TMD and juxtamembrane regions (Figure 3A). While Phe residues in both TMDs proposed to contribute to cholesterol-binding motifs are arranged on the same helical face as the G-XXX-G motifs (Figure 3B), and may be blocked from forming interactions with cholesterol upon tight helix-helix interactions, several of the remaining residues map to the opposite helical face and would thus be exposed to the lipid membrane and available for binding of cholesterol. These residues are similar for both TMDs and are shown for human HLA-DRA1 and

HLA-DRB1 in Figure 3B. It is also interesting to note that these motifs are accessible for cholesterol binding in both MHC-II conformers shown in Figure 3B, although the motif in the  $\beta$  TMD would likely be displaced outside the bilayer and this may impact the binding affinity.

To investigate whether these motifs in the MHC  $\alpha$  and  $\beta$  TMDs directed binding of cholesterol, we used a synthetic peptide construct derived from residues T219-L244 of MHC  $\alpha$  chain and residues M227-I247 of MHC  $\beta$  chain coupled together using Ala-Lys as linkers [83]. Interaction of cholesterol with the synthetic MHC-II TMD was monitored by changes in the fluorescence emission spectrum of 22-NBD-cholesterol, a common fluorescent sterol analog. In the presence of the MHC-II TMD synthetic construct, the fluorescence emission spectrum of NBD-cholesterol underwent a large increase in the intensity of the emission peak, coupled with a blue shift of 40 nm for the emission maximum, indicative of binding of the sterol in a hydrophobic pocket. Cholesterol-dependent conformational changes in the MHC-II TMDs were also monitored using circular dichroism, and revealed that the helical content of the liposome-reconstituted TMDs decreased significantly (from 84% to 68%) in the presence of cholesterol. This result suggests that the binding of cholesterol may unwind the alpha-helical coiled coil formed between the  $\alpha$  and  $\beta$  TMDs, allowing access to the Phe residues on the same helical face as the G-XXX-G motifs and sliding of the two TMDs along one another to stabilize an alternative conformation (for example, Ia.2- vs. Ia.2+ as shown in Figure 3B). This model would be in keeping with the fact that mutation of cholesterol binding residues F240, L243, and F246 in the MHC-II  $\beta$ TMD also decreased the affinity for cholesterol [83]. These binding and

structural studies clearly demonstrated that cholesterol interacts with the MHC-II TMD and alters its conformation [83].

The question still remains as to how a cholesterol induced conformational change in the MHC-II TMD dictates overall conformation of the MHC-II protein in the environment of membrane, and how such changes influence the immune response. At this stage, the relative stabilities of the various MHC-II conformers in the presence and absence of membrane cholesterol remains unknown.

*Impaired MHC-II restricted T-cell activation in a disease model: Role of membrane cholesterol*

We recently reported an unrecognized functional role for MHC-II in the context of membrane lipid environment in a specific disease model, namely visceral leishmaniasis, in which lipid metabolism is altered at the active stage of the disease. Visceral leishmaniasis is caused by the protozoan parasite *Leishmania donovani* (LD) which replicates within macrophages and dendritic cells [84]. The disease is characterized by immune suppression [84], hypocholesterolaemia [85-87] and increased fluidity of infected host cells [77]. Restoration of membrane cholesterol by liposomal cholesterol injection in infected animal models allows expansion of anti-leishmanial T-cells [87] and, interestingly, hypercholesterolaemic mice are solidly protected against lethal challenge of LD infection [88]. During their intracellular life cycle, parasites take up membrane cholesterol which increases membrane fluidity [77]. LD-infected splenic macrophages (LD-macrophage), dendritic cells and/or cells of the monocyte macrophage lineages P388D1 (LD-P388D1) failed to activate T cells

in response to a low dose of exogenous peptide [77]. We also showed that in infected macrophages there is an increase in lateral mobility of membrane proteins [89]; such macrophages are incapable of forming an effective immunological synapse with antigen specific T-cells [90].

The distribution of MHC-II protein ( $A^d$ ) in leishmania infected macrophages was studied to see how an intracellular protozoan parasite like leishmania modulates distribution of MHC-II protein on the cell surface. This was studied in vitro by infecting the monocyte-macrophage cell line (P388D1) with leishmania parasites, and following the intracellular growth as a function of time using microscopy methods. The raft architecture of uninfected P388D1, LD-infected (LD-P388D1) and cholesterol liposomes treated LD-infected P388D1 was studied using raft [CD48 and cholera toxin-B (CTX-B)] and non-raft (CD71)-specific antibodies. Colocalization of CD48/CTX-B and CD71 with raft and non-raft associated MHCII indicated how the distribution of MHCII varied under parasitized conditions and upon treatment of LD infected P388D1 cells with liposomal cholesterol. Binding of anti-CD48 monoclonal antibody (mAb) and CTX-B subunit decreased significantly in LD-P388D1 and, consequently, colocalization with  $A^d$  was not observed, but this was restored in liposomal cholesterol treated LD-P388D1 cells<sup>63</sup>. Conversely, colocalization between  $A^d$  and CD71 remained unaffected regardless of the presence or absence of intracellular LD parasites. Furthermore, uninfected P388D1 and liposome cholesterol treated LD-P388D1, but not LD-P388D1 itself, formed a peptide-dependent synapse with T cells quite efficiently, and this result was supported by intracellular  $Ca^{2+}$  mobilization in T cells and IL-2 production [77].

By exploiting biophysical techniques like surface plasmon resonance spectroscopy (SPR), we determined the kinetic parameters of peptide MHC stability. In this regard p-MHCII complex formation was studied with a limiting concentration of peptide by SPR, while p-MHCII complex formation on the isolated membrane was studied in real time, as described by others [91, 92]. Increasing concentrations of OVA<sub>323-339</sub> (A<sup>d</sup> restricted peptide) were flowed over an immobilized membrane on an L1 chip, and  $k_{on}$  and  $k_{off}$  rates were determined using a Langmuir 1:1 binding isotherm. It was observed that  $k_{on}$  and  $k_{off}$  values were 67.3 ms<sup>-1</sup> and 9.32 ms<sup>-1</sup>, respectively, for normal macrophage membrane, but 5.6 ms<sup>-1</sup> and 32.2 ms<sup>-1</sup>, respectively, for LD-macrophage membranes [93]. This observation indicated that p-MHCII complex formation is compromised in LD-macrophages compared with normal macrophages. This observation has important implications in the immunobiology of visceral leishmaniasis, as patients at the active stage of the disease are immunosuppressed. This may be due to faster dissociation of cognate peptide from the peptide-MHC-II complex leading to a defective anti-leishmanial T-cell response.

#### *Cholesterol driven conformational changes within MHC-II proteins*

Conformational changes within MHC-II during biosynthesis, folding, and in the MHC-II-containing compartment were detected by mAb binding [94-96], which is a powerful tool for this type of study. The 11-5.2 anti-A<sup>k</sup>-mAb recognizes the Ia.2 epitope on A<sup>k</sup> MHC-II, defined as a lipid raft resident MHC-II conformer vital to the initiation of restricted B cell-T-cell activation [94]. Binding of an anti-Ia.2 mAb, such



as 11-5.2, is highly dependent on the residues Arg57 and Gln75 of the A<sup>k</sup> chain, residues near the peptide binding groove [56]. Depletion of membrane cholesterol from APCs by methyl- $\beta$ -cyclodextrin treatment compromised peptide-MHC-II formation coupled with impaired binding of conformational antibody 11.5-2 [83]. The reduced binding of 11.5-2 to Ia2 epitope A<sup>k</sup> could be corrected by restoring membrane cholesterol. Thus membrane cholesterol appears to play an important role in maintaining the active form of MHC-II [83].

Following on from our study demonstrating the interaction of MHC-II TMDs with membrane cholesterol via a CRAC-like motif, which brought about a conformational change in the protein [83], we also investigated how interaction of cholesterol with the TMD influences conformational changes in the distal peptide binding groove of MHC-II. The availability of conformationally and non-conformationally specific anti A<sup>k</sup> mAb greatly aided our investigation. We showed that binding of 11-5.2 to LD infected macrophages (I-M $\Phi$ ) was reduced by about 60% as compared to normal macrophages (N-M $\Phi$ ), whereas binding of 10-2.16 were comparable. The mAb 10-2.16 recognizes the  $\beta$ -chain of A<sup>k</sup>, specifically Ia.17. This observation indicated that A<sup>k</sup> molecules are located on the surface of I-M $\Phi$ , where conformational constraints may limit the binding of 11-5.2 to I-M $\Phi$  when they are not treated with liposomal cholesterol. This effect was cholesterol-specific because the binding of 11-5.2 with I-M $\Phi$  was not restored by cholesterol analogue liposomes (made from 4-cholesten-3-one lacking the OH function of cholesterol) or 1,2 dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) liposome treatment [87, 90].

We used this analogue because the alignment of cholesterol in the membrane is thought to be supported by a hydrogen bond between the cholesterol OH group and the amide bond of sphingolipid [97] and this interaction is presumably not present in the case of this analogue because of the lack of an OH group. To study the importance of cholesterol binding at the appropriate motif on the distal peptide binding groove of MHCII, we mutated putative cholesterol binding residues (F240, L243, and F246 of the  $\beta$  chain) in the TM domain of full-length MHCII (mutant A<sup>k</sup>) and its effects on the nature of the peptide binding groove was monitored using binding of conformation-specific mAb and T cell stimulating ability. CHO cells were transfected with either A<sup>k</sup> or mutant A<sup>k</sup>, which were used as APCs. The binding of conformation-specific antibody was assessed using mAb 11-5.2 and total cell surface expression was assessed using nonconformational antibody (10-3.6). Our study showed that the cell surface expression of MHCII remains unaltered in mutant A<sup>k</sup> whereas binding of conformational antibody was reduced by ~5-fold as compared with CHO cells expressing wild-type MHCII. The ability of transfected cells to stimulate T cells was assayed in terms of IL-2 production in the presence of HEL46-61 peptide. It was observed that mutant A<sup>k</sup> showed compromised ability to stimulate T cell hybridoma (HyH12.6, A<sup>k</sup> restricted) as evident from the decreased IL-2 production as compared with wild-type A<sup>k</sup>. This supports the hypothesis that interaction of cholesterol with the CRAC motif is important to bring about proper conformational orientation. Transfection of CHO cells with wild-type full length MHC-II, but not with mutant MHC-II, activated antigen-specific T cells and decreased the binding of conformation-specific antibodies [83]. Thus, cholesterol-induced conformational changes within

the MHC-II TMD may allosterically modulate the peptide binding groove, leading to T cell activation.

*Analysis of MHC-II-cholesterol interactions by Molecular Dynamics (MD).*

It has been shown through MD simulation studies that cholesterol interacts with the membrane proximal regions of rhodopsin to induce local structural perturbation, which in turn brings global conformational rearrangements through rigid body motion [98]. We performed exhaustive MD simulations to study the effect of cholesterol binding on the structures of the MHC-II TMD, the peptide binding groove, and the protein region linking these two [99]. The results revealed marked variations in flexibility and conformational adaptability of the MHC-II proteins in response to cholesterol binding. Removal of cholesterol from the complex increased the rates of fluctuation of residues along both chains of MHC-II, including those in the peptide binding domain (PBD). Structural alterations in the PBD might lead to loss of peptide binding and/or presentation of peptide to T-cells. Calculation of the solvation free energies ( $\text{Kcal Mol}^{-1}$ ) of the PBD and the bound peptide for a few selected ensemble structures extracted at various time points of the simulation in the presence and absence of bound cholesterol showed significant reductions in energies for most of the complexes in absence of bound cholesterol, indicating that stability of the peptide-MHC-II complex may decrease in the absence of cholesterol (Figure 4).

The MD results provided insight into the mechanistic details of possible long range allosteric adaptation observed for the MHC-II extracellular domain upon cholesterol binding to the TMD. Cross-correlation of flexibility analyses suggest that

changes at the MHC-II TMD are correlated with conformational variation at the PBD [99]. To test the prediction that peptide-MHCII stability is compromised in the absence of cholesterol, we measured kinetic stability on the cell surface. These studies made it clear that the rates of dissociation of the cognate peptide from the complex were 1.45, 16.3, and 1.68  $\mu\text{s}^{-1}$ , respectively, in normal macrophages, infected macrophages, and liposomal cholesterol treated infected macrophages. This was further validated by the progressive decrease in IL-2 production in anti-LACK (Leishmania homolog for the receptor of activated C Kinase) T-cells noted as a function of time. This observation shows a clear association between reduced binding of 11–5.2 to cell surface A<sup>k</sup> and faster dissociation of the cognate peptide from p-MHC-II complex in infected macrophages. The above defects were found to be corrected by liposomal cholesterol treatment of LD-macrophage [93]. On the basis of our computational data coupled with experimental findings, we suggest that the interactions of cholesterol with the MHC-II TMDs may allosterically modify the conformation of the peptide binding groove leading to more stabilization of the p-MHC-II complex on the cell surface. MD based studies involving serotonin 1A [100, 101] and  $\beta$ 2 adrenergic receptors [102] have shown that enrichment of cholesterol within the membrane alters protein oligomerization and stability.

The modulation of cholesterol-induced conformational changes in membrane proteins is complex, and to our knowledge there are no simple methods by which to monitor the nature of changes that alter agonist affinity. However, we were able to show that the reduced p-MHCII stability in cholesterol depleted APCs was restored to normal levels by liposomal cholesterol treatment. Our results bring forth a

mechanistic view to explain how decreased membrane cholesterol in LD-macrophages or in cholesterol depleted APCs may influence conformation and downstream function of MHC-II. Thus liposomal cholesterol may be used as an adjunct with chemotherapy to reduce the dose of chemotherapy required for treatment of visceral leishmaniasis.

To our knowledge, the conformational change of MHC-II in a disease-like condition has only been reported for visceral leishmaniasis, however there are a number of studies that indicate that high cholesterol may protect from infectious diseases [103]. High cholesterol has been linked to protection against a variety of parasitic infections [104] and may have other beneficial effects on the immune system, as suggested in a report showing that hypocholesterolaemic men had significantly fewer circulating lymphocytes, total T cells, helper T-cells and CD8+ cells than hypercholesterolaemic men [105]. Conversely, low levels of cholesterol in critically ill surgical patients have been reported to correlate inversely with concentrations of IL-6, soluble IL-2 receptor and IL-10 [106]. Furthermore, thymic development of immune repertoire is dependent on cholesterol, as the disruption of the lipid rafts of thymic epithelial cells impairs CD4 +T-cells expansion [60].

### **III. Conclusions**

The studies summarized above emphasize the importance of protein-protein and protein-lipid interactions involving the membrane-localized protein regions of the MHC-II, and demonstrate that models of this critical immune receptor should take these regions into account. It has been shown that the TMDs of MHC  $\alpha$ ,  $\beta$  and

invariant chain are the sites of strong and selective interactions that direct correct assembly, transport and function of the complex. These interactions are driven, in part, by well-known TMD helix-helix interaction motifs (e.g. the G-XXX-G motif and motifs of polar residues) shown to impact the folding of a large number of integral membrane proteins. Modification of the TMD sequences of either of the MHC-II proteins or chaperones (e.g. Ii) has been shown to impact assembly and dysregulate transport, leading to retention of the complex in the ER and thus preventing formation of the mature MHC-II required for antigen presentation. We propose that TMD regions are therefore fundamental to the correct function of this immune receptor, and have integrated TMD interactions into the model of MHC-II assembly shown in Figure 2.

Interactions between the TMDs of the MHC-II chains and the membrane have also been shown to impact function. The MHC-II protein needs to be associated with membrane microdomains enriched in sphingolipids and cholesterol (i.e lipid rafts) to induce T-cell activation at a limiting cognate antigen concentration. Both the MHC  $\alpha$  and  $\beta$  chain TMDs contain sequence motifs known to direct the binding of membrane cholesterol (Figure 3A) and we have summarized work that clearly links the presence of cholesterol to maintenance of the correct MHC-II conformation. This apparent requirement for membrane cholesterol may be exploited by the protozoan parasite *Leishmania donovani*, which takes up membrane cholesterol leading to increased membrane fluidity and disruption of membrane rafts. This reduction of membrane cholesterol also causes conformational changes within MHC-II that lead to decreased affinity of cognate antigen, thus contributing to the defective MHC-II restricted T-cell

activation observed in leishmaniasis. Such defects can be corrected by liposomal delivery of cholesterol in leishmania-infected macrophages, indeed liposomal cholesterol treatment offers protection in infected hamsters. This real world example of how a parasite (leishmania) exploits membrane-localized interactions of MHC-II to dampen the hosts' immune response may represent a general strategy of immune system evasion, and underpins the importance of recognizing these regions in any description of MHC-II biology.

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## Figure Legends

**Figure 1. Current Models of Class II MHC (MHC-II).** **A.** In the classical model of Class II assembly in the endoplasmic reticulum (ER), trimers of MHCII-associated invariant chain (Ii) bind three MHC-II alpha/beta heterodimers ( $\alpha/\beta$ ) to form a nine-chain Ii<sub>3</sub> ( $\alpha/\beta$ )<sub>3</sub> complex stabilized by interactions between the CLIP (class-II-associated invariant chain peptide) region in Ii and the MHC-II peptide binding cleft. **B.** An alternative model has also been reported in which each Ii trimer binds a single  $\alpha/\beta$  heterodimer to form a Ii<sub>3</sub>  $\alpha/\beta$  pentamer. Also shown are the residues thought to form the boundaries of the N-terminal cytoplasmic, transmembrane, CLIP and trimerization domains of Ii. **C.** Sequence alignment of the transmembrane domains of the MHC-II invariant chain (CD74) for human, mouse and rat proteins. The transmembrane domain sequence is underlined, and the highly conserved polar amino acids Gln47, Thr49, and Thr50 are shown in bold. The transmembrane domains residues thought to stabilize the Ii trimer are highlighted using asterisks.

**Figure 2. Extended model for MHC-II assembly, accounting for contributions of transmembrane regions.** **A.** Previous data suggest that the strong and specific interactions observed between Ii TMDs may initiate Ii trimer formation and direct optimal orientation of the luminal domains. **B.** Although not reported to date, similar interactions between the TMDs of Ii and MHC-II molecules may also contribute to complex formation. **C.** Such interactions have also been observed between the TMDs of MHC  $\alpha$  and  $\beta$  chains, and mutation of TMD residues involved in these interactions has been shown to impact MHC-II function.

**Figure 3. Transmembrane helix–interaction and cholesterol binding motifs in MHC-II TMDs lead to different conformations.** **A.** Alignment of the putative TMD sequences of human, mouse and rat MHC-II  $\alpha$  and  $\beta$  chains, with corresponding sequence IDs and residue numbers given. In each sequence, the TMD is underlined, whilst additional C-terminal juxtamembrane residues are also shown. Conserved G–XXX–G helix-helix interaction motifs in both chains are highlighted in light gray, and conserved cholesterol-recognition motifs are shown in bold. **B.** The differential pairing of G–XXX–G motifs in the  $\alpha$  and  $\beta$  chains (shown as Gly residues linked via a bracket) is proposed to give rise to two distinct MHC-II conformers, residing in different membrane environments and having differing abilities to stimulate T-cells. The relative position of residues within the cholesterol-recognition motifs in the helix is also shown.

**Figure 4. Cholesterol binding to the MHC-II TMDs may destabilize peptide binding.** Computational and experimental evidence suggest that the stability of peptide-MHC-II complexes decreases in the absence of membrane cholesterol, possibly through direct interactions between cholesterol and TMDs. This is thought to occur through cholesterol-induced conformational changes in the MHC-II TMD that allosterically modulate the peptide binding groove, leading to optimal peptide binding and downstream T cell activation.



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